

# Cell Factory Engineering Using Combinatorial and *In Vitro* Evolution Strategies

## NIH-Grant: "Biosynthesis of Unnatural Porphyrins"

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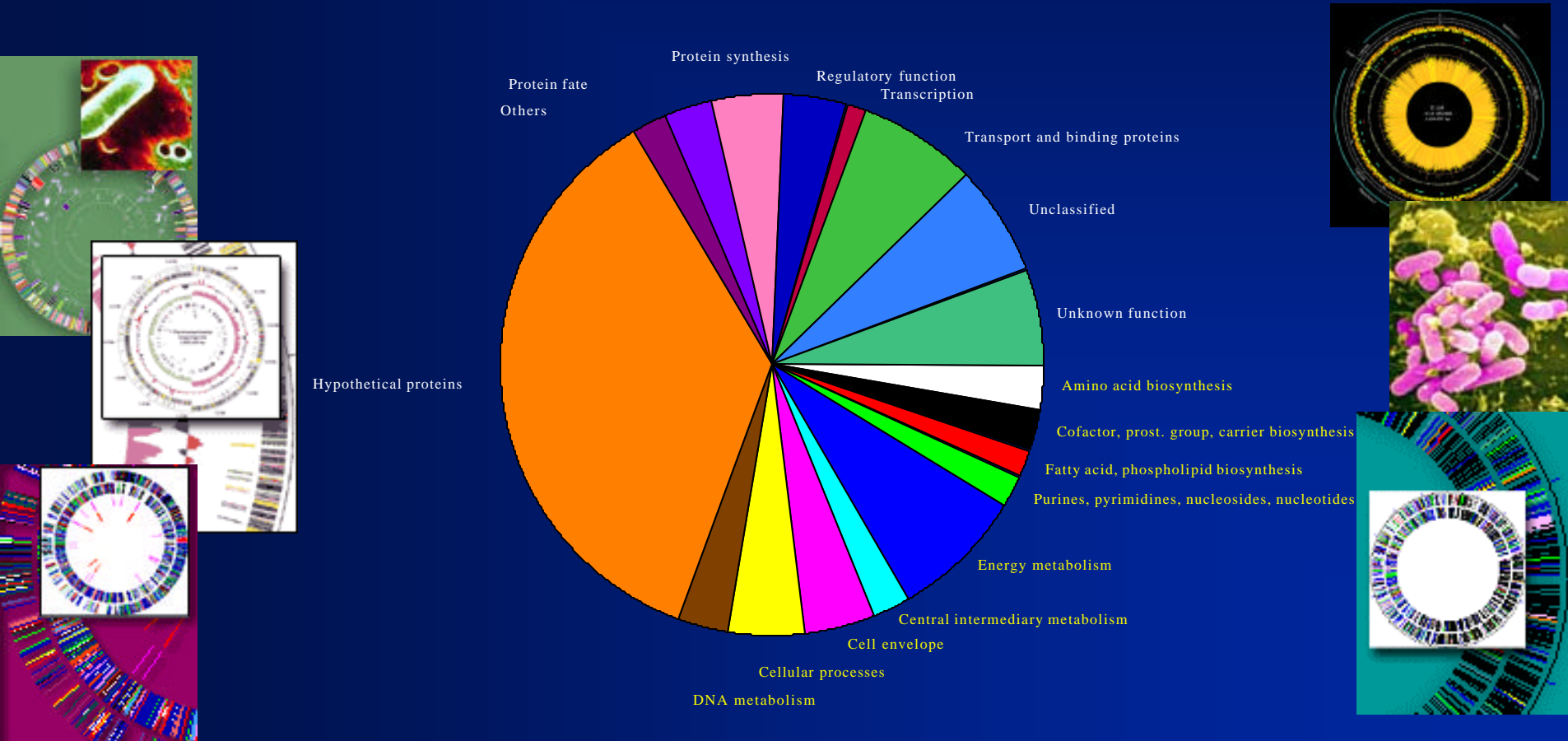
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# Cell Factory Engineering Using Combinatorial and *In Vitro* Evolution Strategies

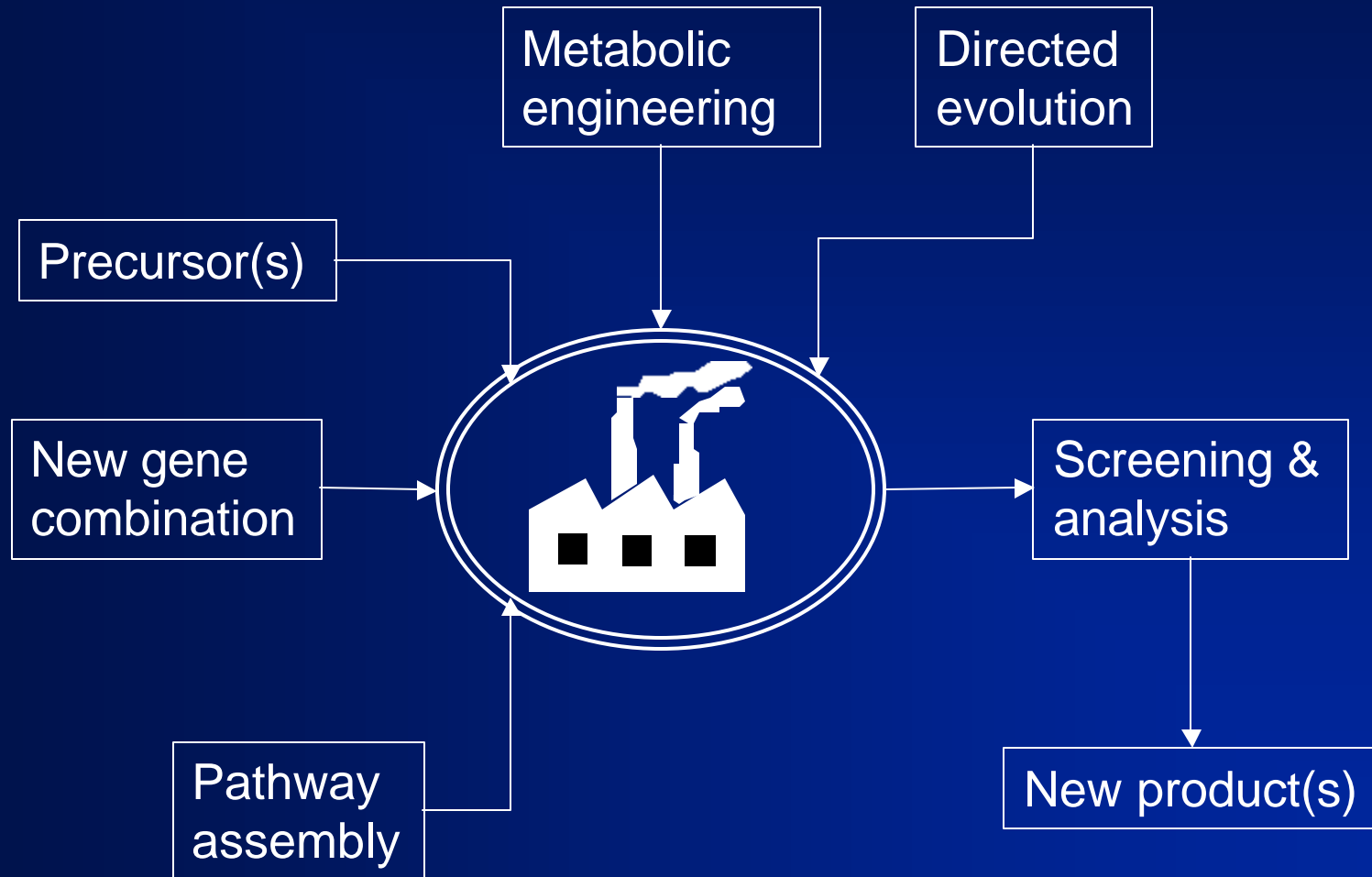
# Genomics – Toolbox of metabolic genes

~ 400 microbial genomes completed or in progress are listed @ GOLD & TIGR DB

**Out of 248,825 genes @ TIGR DB, ~ 30% of the known genes encode metabolic enzymes**

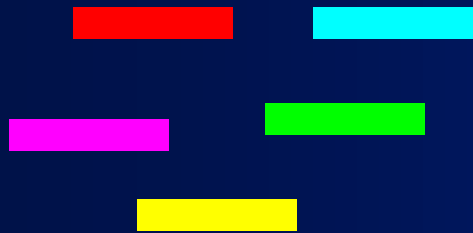


# Strategies for cell factory engineering

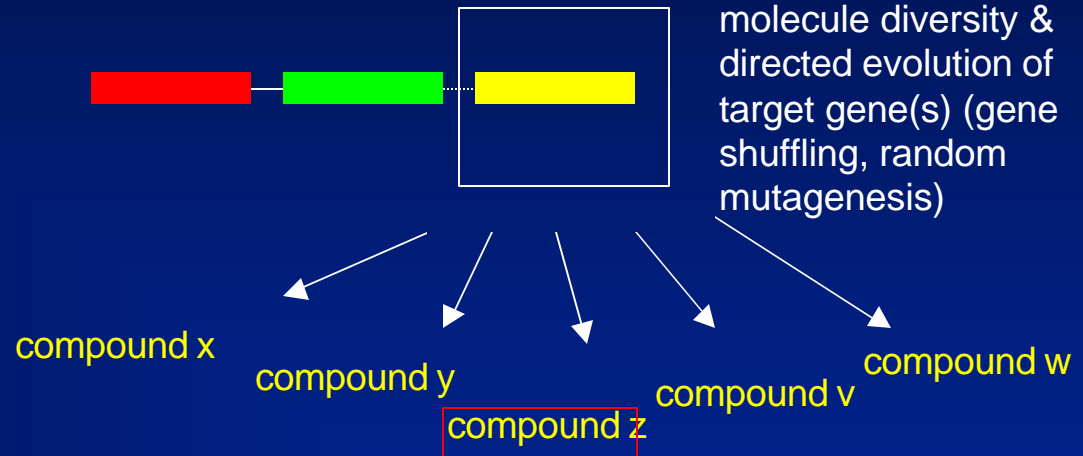


# *In vitro* evolution of pathways

Set of selected biosynthetic genes

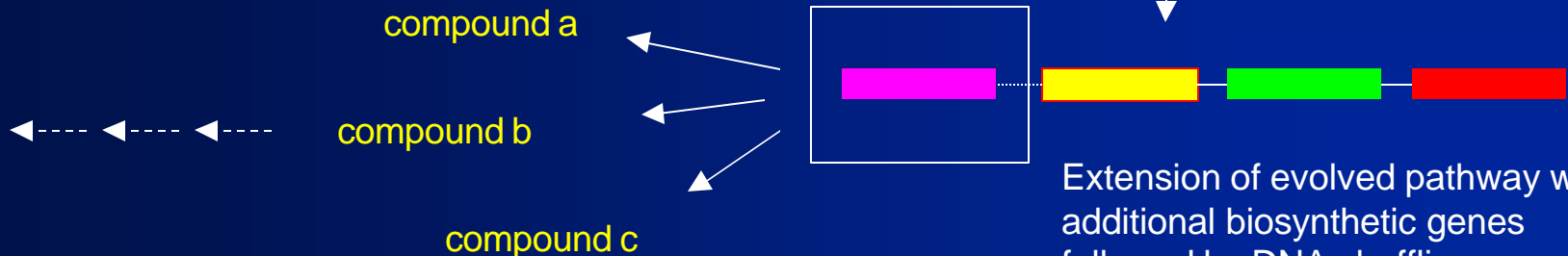


Pathway assembly



Identification of key enzyme(s) for molecule diversity & directed evolution of target gene(s) (gene shuffling, random mutagenesis)

Extension of evolved pathway with additional biosynthetic genes followed by DNA shuffling or random mutagenesis of gene(s)



# Biosynthesis of novel carotenoid structures in *E. coli* using combinatorial and *in vitro* evolution strategies.

## Acknowledgments

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# Carotenoids



## Functions

- ❖ coloration
- ❖ photosynthesis and photoprotection
- ❖ in nutrition and health: vitamin A and retinoids  
antioxidants, tumor suppressing, prevention of chronic diseases  
specific tumor suppressing activities

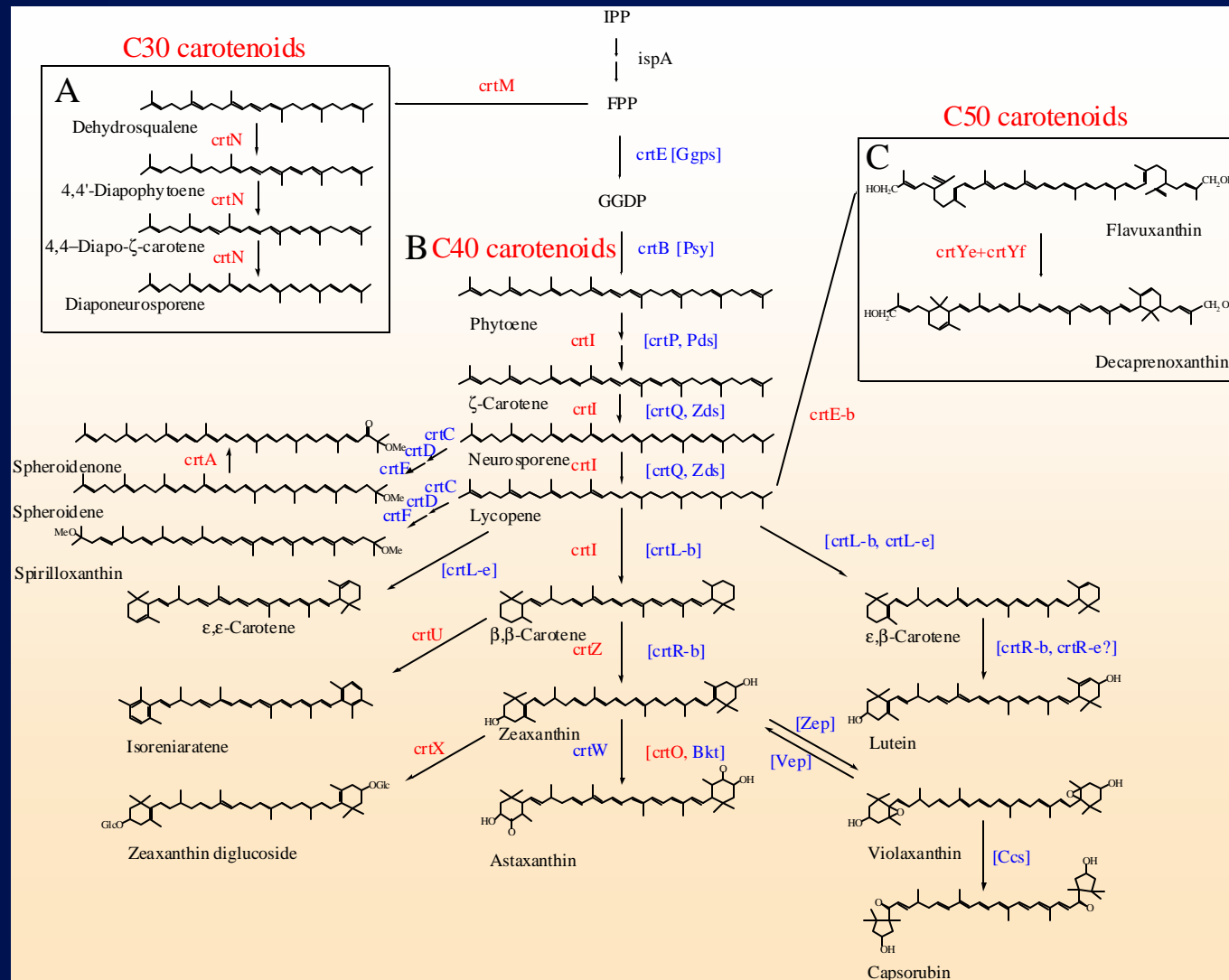


## Industrial importance

- ❖ food colorants
- ❖ animal feed supplements, e.g. aquaculture of salmon, poultry industry
- ❖ antioxidants
- ❖ vitamin A precursors



# Carotenoid biosynthesis



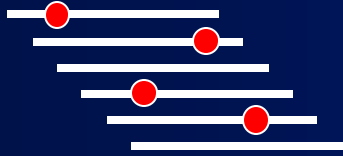


# Carotenoid gene toolbox

Gene	Enzyme	Catalyzed reaction	Strain
<b>Isoprenoid precursor biosynthesis</b>			
<i>crtE</i>	GGDP synthase	head-to-tail condensation of IDP + FDP	<i>Erwinia uredevora</i>
<i>ispA</i>	FDP synthase	head-to-tail condensation of 2 IDP + DMADP	<i>E. coli</i>
<i>fgs</i>	FGDP synthase	head-to-tail condensation of 3 IDP + DMADP	<i>Aeropyrum pernix</i>
<b>Generation of C40 carotenoid backbone</b>			
<i>crtB</i>	Phytoene synthase	head-to-head condensation of 2 GGDP	<i>Erwinia uredevora</i>
<i>crtI</i>	Phytoene desaturase	introduction of 4 desaturations in phytoene	<i>Erwinia uredevora</i> <i>Erwinia herbicola</i>
<b>Generation of C30 carotenoid backbone</b>			
<i>crtM</i>	Dehydrosqualene synthase	head-to-head condensation of 2 FDP	<i>Staphylococcus aureus</i>
<i>crtN</i>	Dehydrosqualene desaturase	introduction of 3 desaturations in dehydrosqualene	<i>Staphylococcus aureus</i>
<b>Modification of C40 carotenoid end-group</b>			
<i>crtY</i>	Lycopene cyclase	cyclization of $\Psi$ end groups in lycopene to $\beta$ end groups	<i>Erwinia uredevora</i> <i>Erwinia herbicola</i>
<i>crtE -b</i>	Lycopene elongase	transfer of 2 C5 isoprenoid units to lycopene	<i>C. glutamicum</i>
<i>crtYe/Yf</i>	Heterodimeric carotene cyclase	cyclization of C45 and C50 carotenoids	<i>C. glutamicum</i>
<i>crtA</i>	Neurosporene monooxygenase	oxygenation at C2 of neurosporene	<i>Rhodobacter sphaeroides</i> <i>Rhodobacter capsulatus</i>
<i>crtO</i>	Carotene oxygenase	oxygenation at C4 of $\beta$ -carotene	<i>Synechocystis sp.</i>
<i>crtU</i>	Carotene desaturase	desaturation/methyltransferation of $\beta$ rings in $\beta$ -carotene (aromatic ring formation)	<i>Streptomyces griseus</i> <i>Brevibacterium linens</i>
<i>crtZ</i>	$\beta$ -carotene hydroxylase	hydroxylation at C3 of $\beta$ -carotene	<i>Erwinia uredevora</i> <i>Erwinia herbicola</i>
<i>crtX</i>	zeaxanthin glucosylase	glycosylation at C3 of zeaxanthin	<i>Erwinia uredevora</i> <i>Erwinia herbicola</i>

# Pathway evolution strategy

Generation of variant library



Liagation & transformation



Filter transfer of colonies & visual screening

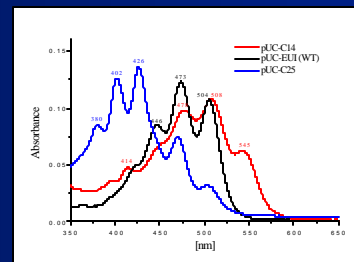


Cultivation of library

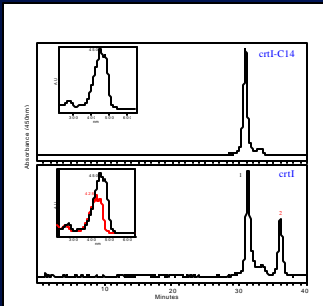


UV/Vis – screening

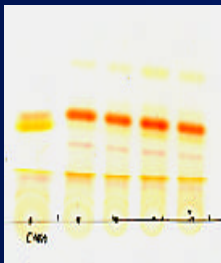
Carotenoid extraction



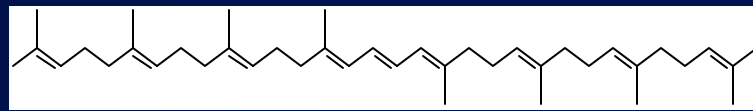
HPLC-UV/Vis or  
HPLC-UV/Vis-MS



TLC –  
screening

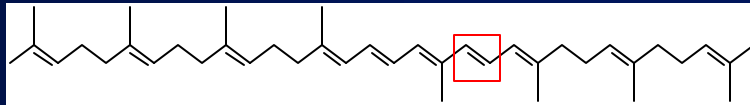


# Introduction of novel branch points upstream in assembled pathway by *in vitro* evolution



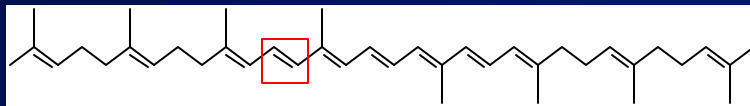
Phytoene

crtI



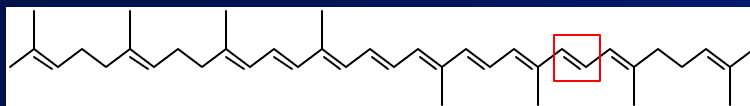
Phytofluene

crtI



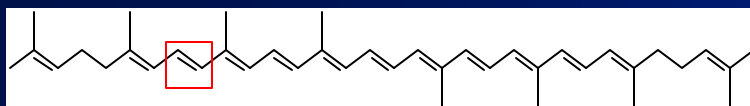
z-Carotene

crtI



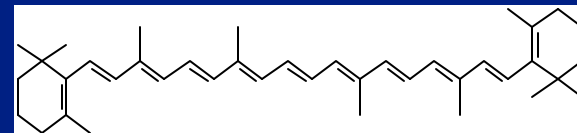
Neurosporene

crtI

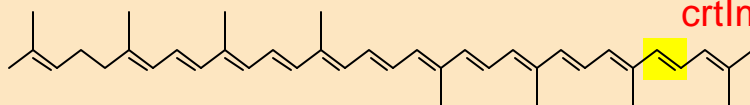


Lycopene

crtY

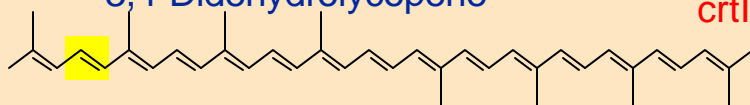


$\beta,\beta$ -Carotene



3,4-Didehydrolycopene

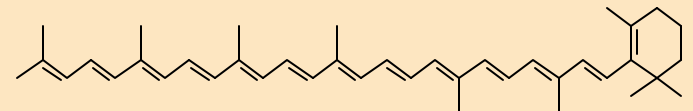
crtImut



3,4,3',4'-Tetrahydrolycopene

crtImut

crtYmut



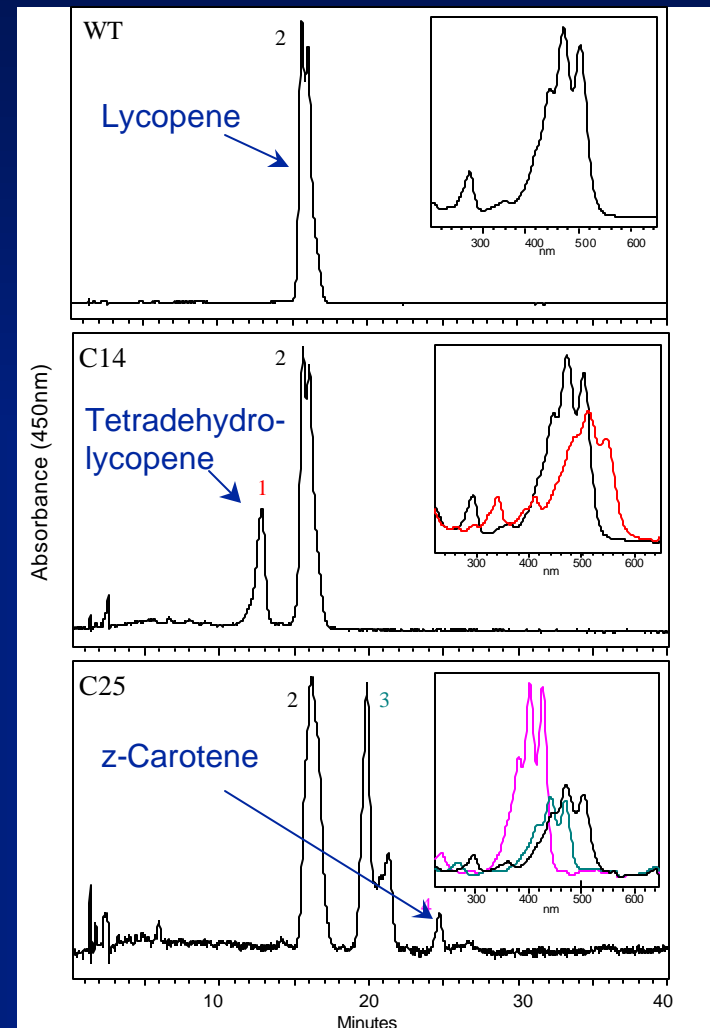
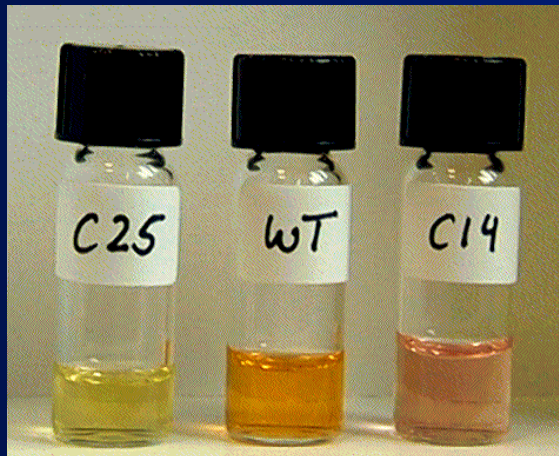
Torulene

# Extension of central carotenoid desaturation pathway by *in vitro* evolution of desaturase crtI

Library (~10<sup>4</sup> clones) of shuffled *crtI* genes yielded:

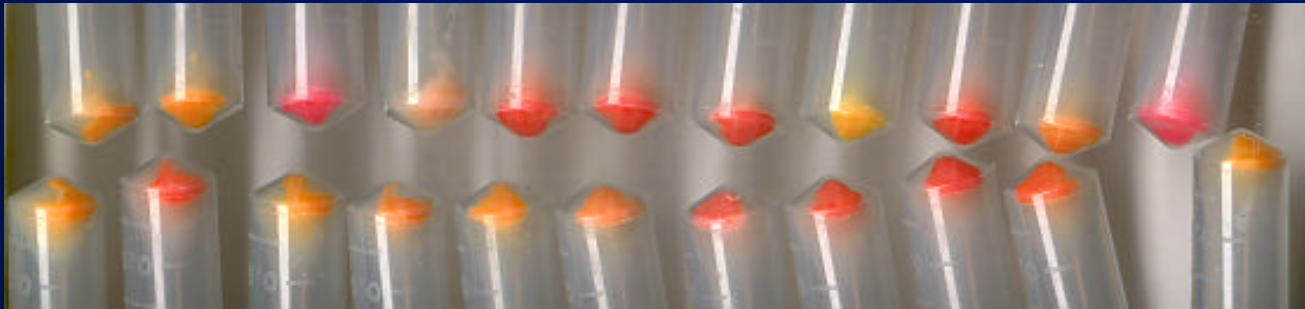
1-2 yellow mutants per 500 clones (C25 selected)

1 pink mutant (C14)

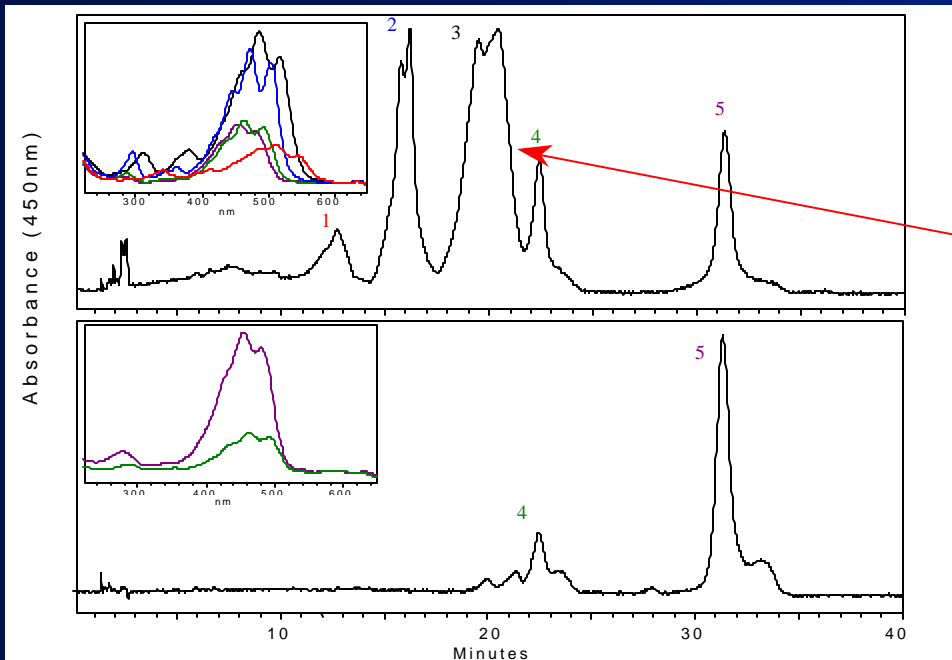


# Extension of evolved desaturase pathway with a library shuffled lycopene cyclases crtY

Y14



Y2

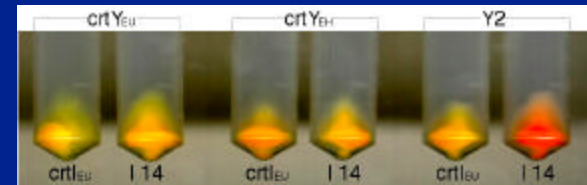


## C14 + Y2

- 1 Tetrahydrolycopene
- 2 Lycopene
- 3 **Torulene**
- 4  $\beta$ , $\gamma$ -Carotene
- 5  $\beta$ , $\beta$ -Carotene

## crtl + Y2

- 4  $\beta$ , $\gamma$ -Carotene
- 5  $\beta$ , $\beta$ -Carotene



# Biosynthesis of unnatural porphyrins in *E. coli*

## Acknowledgements

Arjo de Boer

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NIH #GN65471

# Specific Aims

- Aim 1:** Establish porphyrin overproduction in *E. coli* by cloning of metabolic genes from different bacteria and assembly into functional biosynthetic pathways.
- Aim 2:** Establish and develop strategies for quantitative and qualitative porphyrin analysis and high-throughput screening of *E. coli* libraries.
- Aim 3:** Create new metabolic pathways for the production of structurally diverse unnatural porphyrins by molecular breeding.

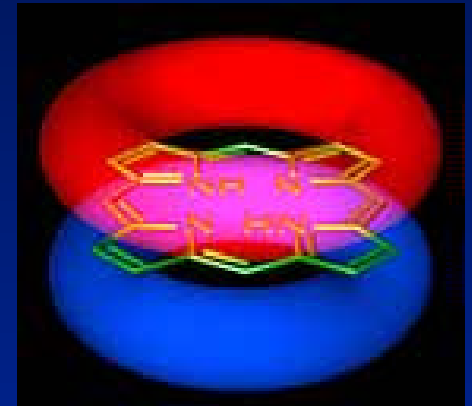
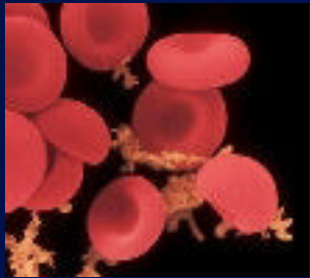
Project I: Expanded porphyrins

Project II: Unnatural modified tetrapyrrolic porphyrins

# Porphyrins

## Functions

- ❖ electron transport systems
- ❖ prosthetic groups
- ❖ “pigments of life” or “colors of life”



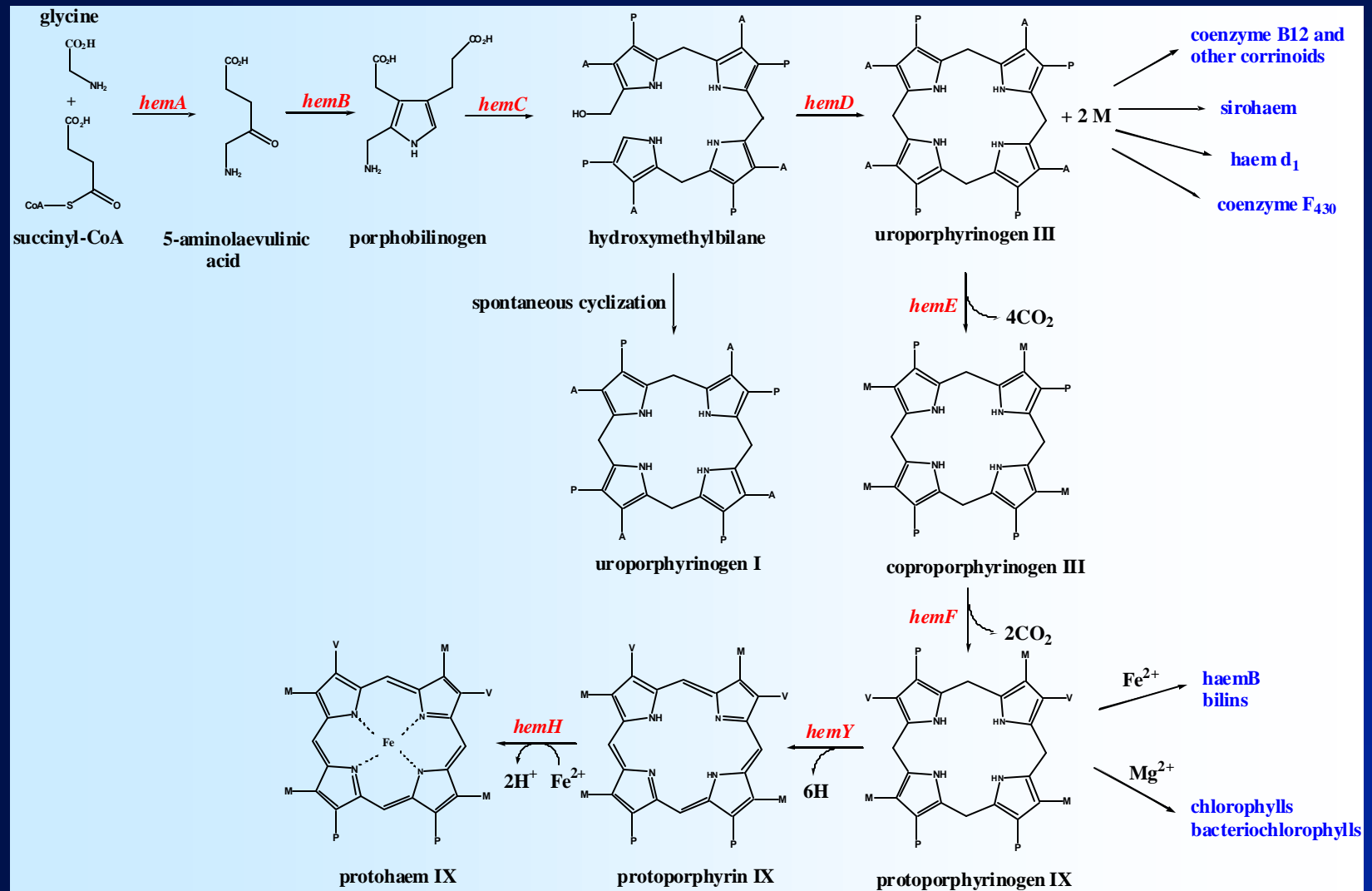
## Applications

- ❖ photodynamic treatment of cancer (laser dyes)
- ❖ various biomedical applications: treatment of viruses, gene regulation therapies, drug targeting
- ❖ material sciences – e.g. porphyrin arrays
- ❖ chemistry – e.g. electro catalysis, electrodes in fuel cells





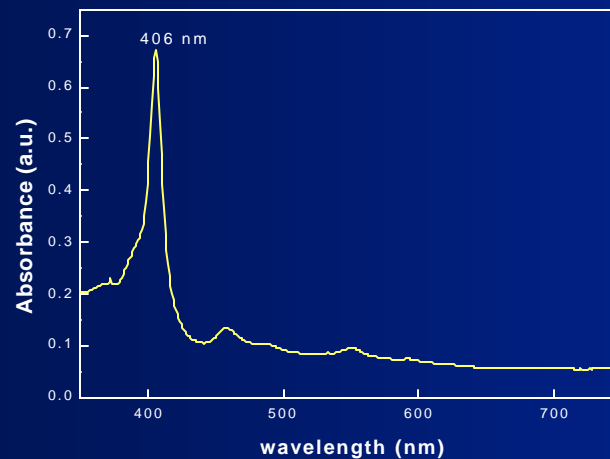
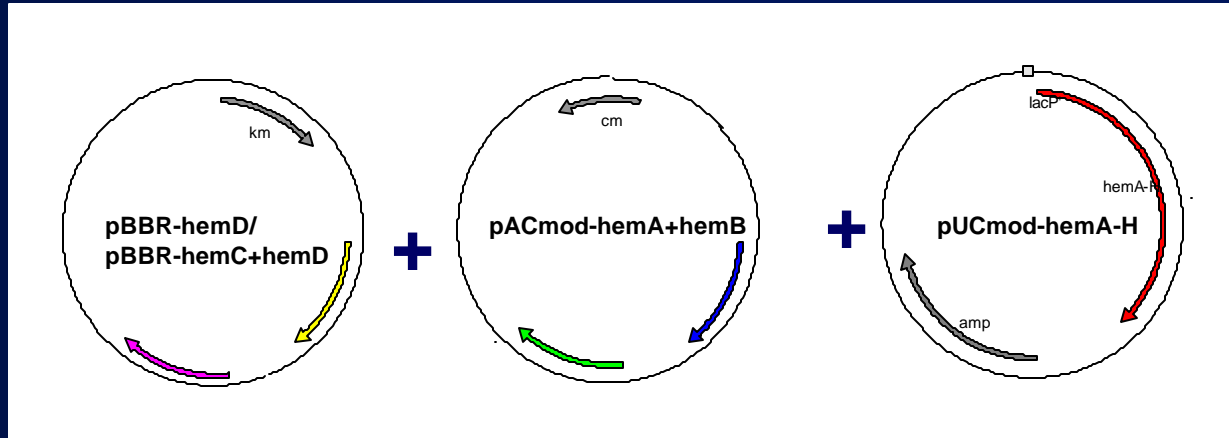
# Porphyrin biosynthesis



# Porphyrin gene toolbox

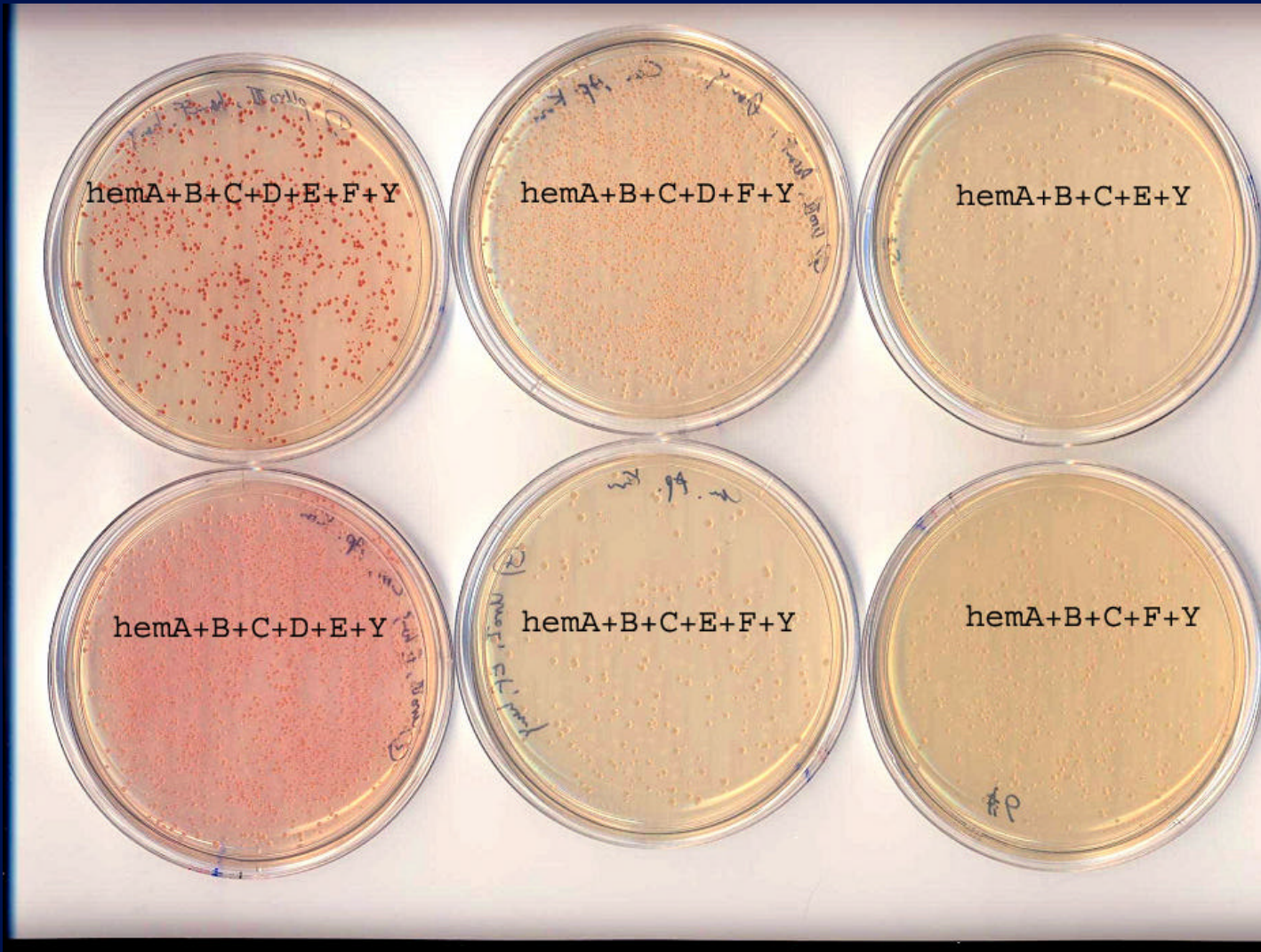
Gene	Enzyme	Catalyzed reaction	Strain
<i>hemA</i>	ALA-synthase	condensation of succinyl CoA + glycine	<i>R. capsulatus</i>
<i>hemB</i>	ALA-dehydratase	dimerization of 2 ALA to form porphobilinogen	<i>E. coli</i>
<i>hemC</i>	porphobilinogen deaminase	deamination and polymerization of 4 porphobilinogens to form the unstable hydroxymethylbilane	<i>E. coli</i> <i>Synechocystis sp.</i> <i>R. capsulatus</i> <i>S. typhimurium</i> <i>K. pneumoniae</i>
<i>hemD</i>	uroporphyrinogen III synthase	ring D inversion + cyclization of hydroxymethylbilane to uroporphyr-inogen III (uro'gen III)	<i>E. coli</i>
<i>hemE</i>	uroporphyrinogen decarboxylase	decarboxylation of 4 acetate side-chains of uro'gen III to form coproporphyrinogen III (copro'gen III)	<i>E. coli</i> <i>Synechocystis sp.</i> <i>R. capsulatus</i>
<i>hemF</i>	coproporphyrinogen III oxidase	oxidative decarboxylation of two propionate side chains in rings A + B of copro'gen III to vinyl groups to form protoporphyrinogen IX (proto'gen IX)	<i>E. coli</i> <i>Synechocystis sp.</i>
<i>hemY</i>	protoporphyrinogen IX oxidase	six-electron oxidation of proto'gen IX to form protoporphyrin IX	<i>B. subtilis</i> <i>B. halodurans</i>
<i>hemH</i>	ferrochelatase	Fe <sup>2+</sup> -metallation of protoporphyrin IX to produce protohaem IX	<i>E. coli</i> <i>R. capsulatus</i> <i>B. subtilis</i>

# Porphyrin pathway assembly

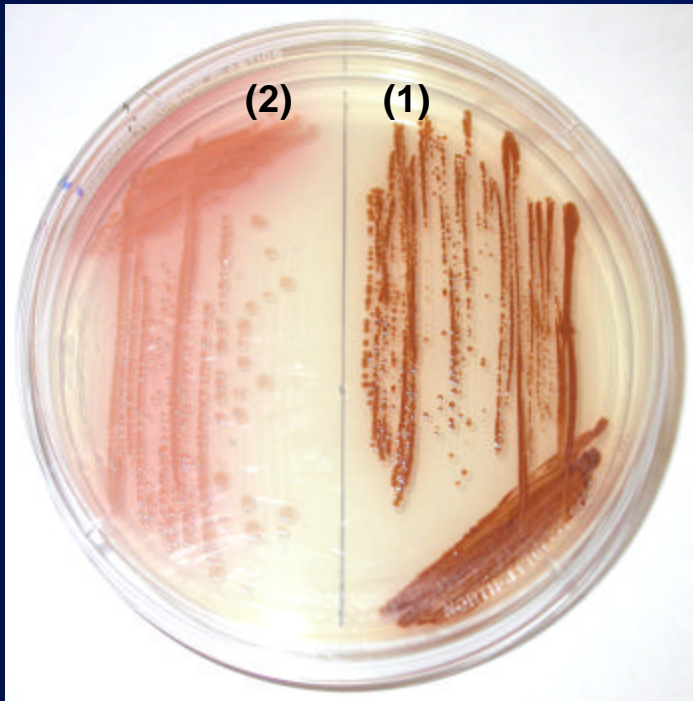


Uroporphyrin I overproduction in *E. coli*

# Reconstruction of porphyrin pathways in *E. coli*

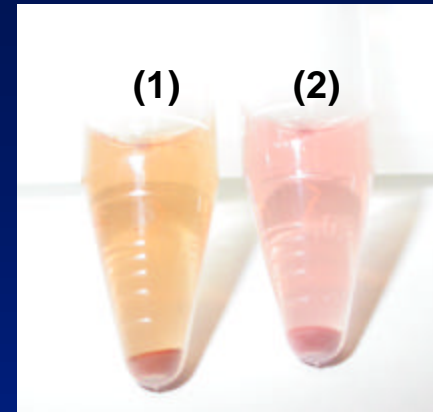


# Protoporphyrin production in recombinant *E. coli*

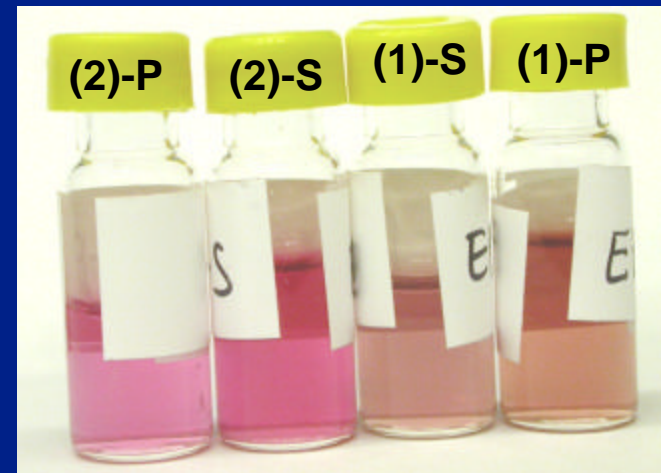


(1) hemA+B+C+D+E+F+Y  
(2) hemA+B+C+D+E+Y

centrifugation



Extraction  
(acetone:HCl 10:1)



Extracts from cell pellets (P)  
and culture supernatants (S).

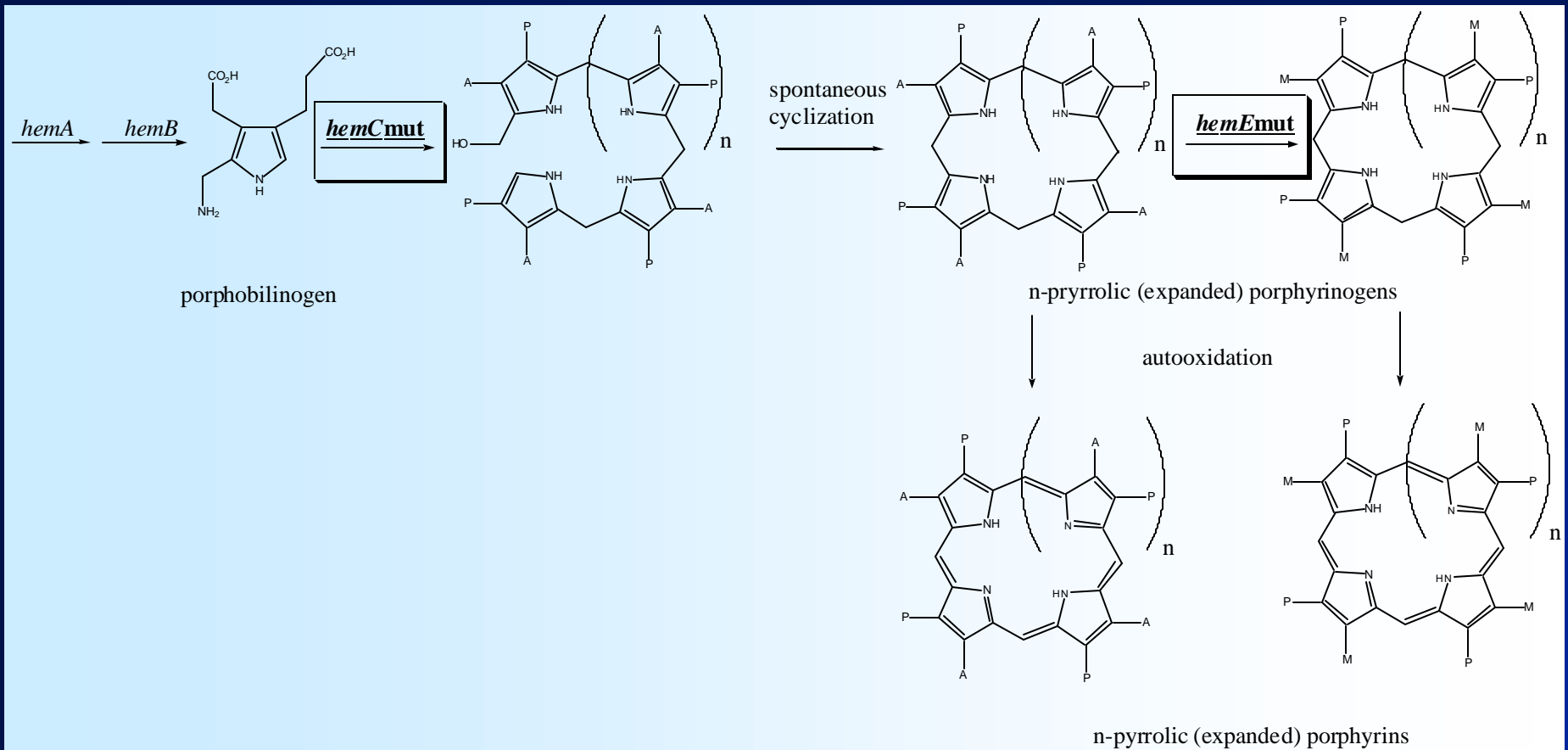


# Microbial production levels of vitamin B<sub>12</sub>

Species of micro-organism or microbiological process	Main component of culture medium	Conditions of fermentation	Vitamin B <sub>12</sub> production (mg/l)
<i>Propionibacterium freudenreichii</i>	Glucose	Anaerobiosis, 5,6-dimethyl benzimidazole	206.0
<i>Rhodopseudomonas protamicus</i>	Glucose	5,6-dimethyl benzimidazole	135.0
<i>Propionibacterium shermanii</i>	Glucose	5,6-dimethyl benzimidazole	60.0
<i>Pseudomonas denitrificans</i> *	Sucrose	Aerobiosis, betaine	60.0 *
<i>Nocardia rugosa</i>	Glucose	Aerobiosis	18.0
<i>Rhizobium cobalaminogenum</i>	Sucrose	Aerobiosis	16.5
<i>Micromonospora</i> sp.	Glucose	5,6-dimethyl benzimidazole	11.5
<i>Streptomyces olivaceus</i>	Glucose	5,6-dimethyl benzimidazole	6.0
<i>Nocardia gardneri</i>	Hexadecane	Aerobiosis	4.5
<i>Butyribacterium methylotrophicum</i>	Methanol	Anaerobiosis	3.6
<i>Pseudomonas</i> sp.	Methanol	5,6-dimethyl benzimidazole	3.2
<i>Arthrobacter hyalinus</i>	Isopropanol	5,6-dimethyl benzimidazole	1.1

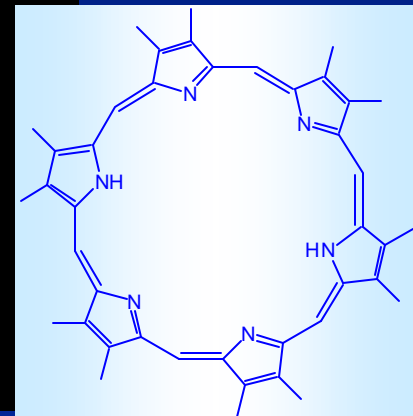
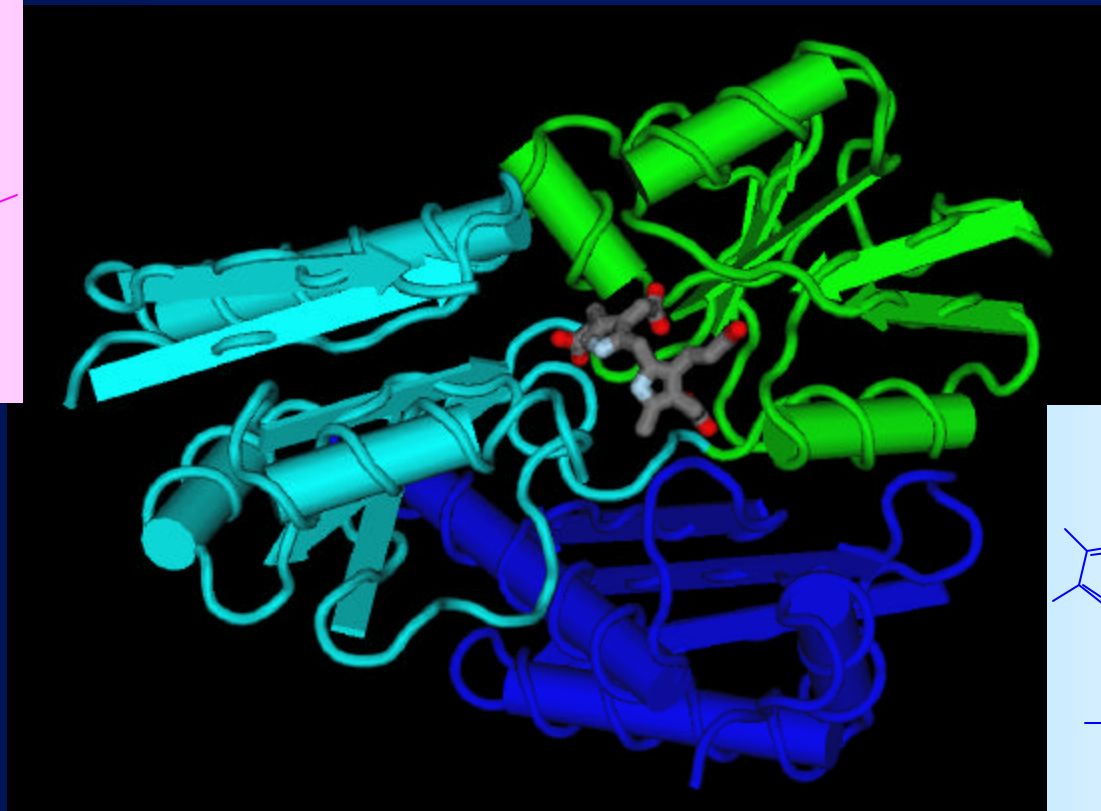
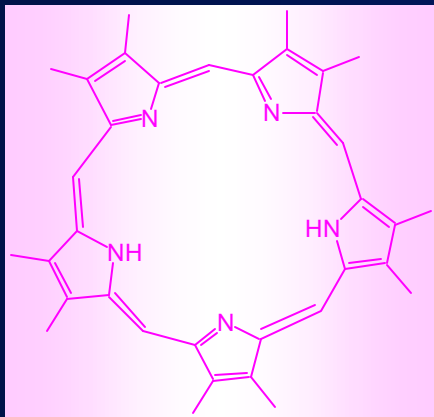
\* Rhône Poulenc Rorer uses a genetically engineered strain supposedly producing 100-300 mg/l

# Biosynthesis of expanded porphyrins



# Biosynthesis of expanded porphyrins

Increasing the porphobilinogen polymer chain length by enlarging the active site cleft through *in vitro* evolution.



Hydroxymethylbilane synthase HMBS (hemC) from *E. coli*



## Next steps

### Project 1: Expanded porphyrins

- ❑ analyze the products formed by hemC
- ❑ investigate the role of the dipyrromethane co-factor
- ❑ if unnatural oligopyrrole products are synthesized, we will further optimize oligopyrrole assembly scaffolds by using *in vitro* evolution and/or rational approaches

### Project 2: Unnatural tetrapyrroles

- ❑ further diversification of tetrapyrrole synthesis in *E. coli* through directed evolution of hemF to obtain variants with novel substrate and product specificities.
- ❑ investigate and identify rate limiting step of metal-porphyrin synthesis in *E. coli* e.g. overexpression of metal-uptake transporter
- ❑ analyse *in vivo* chelatase activity of hemH - directed evolution of hemH to obtain variants with novel chelatase activities

Further develop and refine high-throughput screening methods using HT-TLC.